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简 报

Glucose Metabolism During Kunming Mouse Preimplantation Development: Analysis of Gene Transcription in Embryos *in Vivo*

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Abstract: In order to investigate glucose metabolism pathways and their changes in Kunming mouse preimplantation 1-, 2-, 4-, 8-cell, and morula embryos, the mRNA level for the genes involved in glucose metabolism was tested by nested RT-PCR on embryos at different development stages *in vivo*. These genes were glucose 6-phosphate dehydrogenase (G6PDH), phospho-fructokinase (PFK), and phosphoglucomutase (PGM), representing pentose phosphate pathway (PPP), glycolysis, and glycogensis and glycogenolysis respectively. Three sets of inner and outer primers were designed and synthesized based on cDNA sequences of G6PDH, PFK and PGM. RT-PCR results revealed that G6PDH gene transcription was found in Kunming mouse 1 – 8 cell embryos, and not in morula embryos; it indicated that 1 – 8 cell embryos may metabolize glucose by pentose phosphate pathway, but morula embryos can not do so. PFK gene transcription was found in 1 – 8 cell and morula embryos; it is probable that there exists glycolysis in those embryos. PGM gene transcription was not found in 1 – 8 cell and morula embryos, so glycogenesis and glycogenolysis in these embryos were not present.

Key words: Mouse embryo; RT-PCR; Gene transcription; Glucose metabolism

The blocks of embryo development *in vitro* are observed in mouses (2-cell stage), cattle, sheep (8 – 16 cell stage) and swine (4-cell stage) (Bavister, 1988). Some researchers found that the development of most mammalian early embryos *in vitro* was hampered in the presence of glucose (Schini & Bavister, 1988; Barneet & Bavister, 1996); others found that it was not (Scott & Whittingham, 1996; Summers *et al*., 1995). An effect of glucose to the early embryo development *in vitro* is contradictive and needs to be studied.

During early preimplantation stages, mammalian embryos do not metabolize much glucose, and energy of cleavage stages is produced by pyruvic acid on TCA pathway (Leese & Barton, 1984; Gott *et al*., 1990; Lane & Gardner, 2000), later which may be required to support normal morula-blastocyst transition (Gott *et al*., 1990; Brison & Leese, 1991; Khurana & Niemann, 2000) and changes into lactic acid (Gardner & Leese, 1990).

Enzymatic studies in mouse, bovine and human embryos reveal that the switch to glucose utilization involves mainly 6-phosphofructokinase (PFK) (Barbehenn *et al*., 1974), glucose 6-phosphate dehydrogenase (G6PDH) (Lequarre *et al*., 1997) and phosphogluco-mutase (PGM). The amount of enzyme synthesized depends on the level of transcription, the stability of message RNA, and the rate of translation. The activity of protein also may depend on posttranscriptional regulation (Lequarre *et al*., 1997).

The purpose of the work was to study the regulation of the transcription for several enzymes involved in

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glucose metabolism of Kunming mouse embryos *in vivo* according to their developmental stages, to analyse the mRNA transcription of these enzymes, to speculate on the pathways of the glucose metabolism and switch in mouse early embryos, and to try to find the effects of glucose on blocks of the early embryo development *in vitro*.

1 Materials and Methods

1.1 Embryo production

Kunming female mice were superovulated by intraperitoneal injections of pregnant mare serum gonadotropin (PMSG, 15 IU), following by an intraperitoneal injection of human chorionic gonadotropin (hCG, 15 IU) 48 h later, and then were immediately mated with Kunming male mice. The females are a random-bred strain which blocks at the 2-cell stage in many media.

Embryos that developed *in vivo* were isolated from superovulated females at 25 – 27 h (1-cell), 44 – 48 h (2-cell), 56 – 60 h (4-cell), 68 – 72 h (8-cell), and 96 – 100 h (morula) after hCG injection and mating with males. Embryos were flushed from the reproductive tract in physiological saline solution and hyaluronidase treated to remove cumulus cells (if necessary), washed in physiological saline solution to remove debris, and frozen 125 (1-cell), 84 (2-cell), 75 (4-cell), 80 (8-cell), 121 (morula) embryo aliquots at −70℃ until processed for RNA.

1.2 RNA extraction and reverse transcription

Total RNA was isolated from the mouse liver and from 1-, 2-, 4-, 8-cell, and morula-stage embryos that had developed *in vivo* using the acid guanidinium phenol chloroform (AGPC) method of Chomsczynski & Sacchi (1987) as modified by Gaudette *et al.* (1993). The RNA pellet obtained after centrifugation was washed with 75% ethanol, then briefly dried before being dissolved in 5 μL of deionized DEPC-treated water.

To unravel RNA secondary structure, samples were heated at 90℃ for 5 min, then quickly chilled on ice, and immediatedly reverse-transcribed. Reverse transcription was performed at 37℃ for 1 h in a 10 μL volume containing 50 mmol/L Tris-HCl, pH8.3, 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 0.5 mmol/L of each dNTP, 0.75 μmol/L downer primer, 10 mmol/L DTT, 200 U RNasin, and 100 U MMLV reverse transcriptase (Gibco-BRL). After reverse transcription, the volume of each sample was increased to 20 μL.

1.3 Multiplex PCR protocol

1.3.1 Primers Primer pairs were designed by our lab and synthesized by Sangon Company in Shanghai. All the primers are listed in Table 1.

1.3.2 Amplification All thermal cycling steps were carried out in Amplitron Ⅱ and in 0.2 mL MicroAmp reaction tubes. Twenty microliters of reverse transcription production from the mouse liver, 1-, 2-, 4-, 8-cell, and morula-stage embryos were added to control and test tubes, resulting in a final concentration of 10 mmol/L Tris-HCl, pH8.3 with 0.15 μmol/L of outer upper primers, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.1 mmol/L dNTPs, and 2 U Taq polymerase in total

Table 1 Primers Used for PCR

Gene		Primer sequence	Annealing temperature (℃)	Reference	DNA Size (bp)
G6PDH	Outer	5′ – GTGAACGTGTTTGGCAGCGGCAAC – 3′	60	Mouse	726
	Primer	5′ – TGTCTCGATTCCAGATGGGTCCAA – 3′		(Zollo *et al.*, 1993)	
	Inner	5′ – CATCATGGGTGCATCGGGTGACCT – 3′	58		378
	Primer	5′ – TGACTCATGCAGGTCTCTTGAATG – 3′			
PFK	Outer	5′ – GCTACCGTGGACCTGGAGAAACTGC – 3′	62	Mouse	881
	Primer	5′ – ACAGTCACTCGTGTATCGAAGCCCA – 3′		(Gehnrich *et al.*, 1988)	
	Inner	5′ – CTGACCAGCGGCGGTGATGCGCA – 3′	59		676
	Primer	5′ – TCTCACACATGAAGTTCTCCCAG – 3′			
PGM	Outer	5′ – GTGAAGATCGTGACAGTTAAGAC – 3′	56	Human	1076
	Primer	5′ – TTCCAGCCAGTTGGGGTCTCATA – 3′		(Whitehouse *et al.*, 1992)	
	Inner	5′ – GAACTTCATCCAGAGTATCATCT – 3′	54		617
	Primer	5′ – CACCGAGTTCTTCACAGAGGATC – 3′			

volume of 100 μL. Tube contents were briefly vortexed. DNA was denatured at 94℃ for 2 min, and PCR was conducted in 30 cycles of 45 s at 94℃ for DNA denaturation, 45 s at different temperatues for annealing of primers (Table 1), 60 s at 72℃ for primer extension. A final extension step of 10 min at 72℃ ended the reaction. Twenty microliters of amplified DNA were transferred to a new reaction tube containing 30 μL of master mix (see above) supplemented with 0.3 μmol/L of inner primers and thermal cycled as described. Fifteen microliters of amplified DNA were electrophoresed for 20 min at 100 V through a 3% agarose containing 0.5 μg/mL ethiduium bromide. Bands were visualized with an UV transilluminator and photographed.

2 Results and Discussion

2.1 G6PDH

Total RNA was extracted with the technique of RT-PCR from the mouse embryos, which developed to the appropriate stage *in vivo*. In order to detect RT-PCR product, a nested PCR protocol was employed in which cDNA from the mouse embryos was subjected to two sets of PCR amplification with an outer primer pair followed by an inner primer pair. Initially, cDNA was tested with primers for G6PDH to confirm successful the RNA isolation and reverse transcription. The expected amplification product for G6PDH was detected in mRNA from 1-, 2-, 4-, 8-cell embryos, as well as in mouse liver, used as positive control, but not in morula (Fig.1). It indicated there is the transcription of G6PDH gene in 1 – 8 cell embryos.

Glucose-6-phosphate can be transformed into glycogen, and metabolized through the pentose-phosphate pathway (PPP) or glycolysis probably. We have investigated and detected the G6PDH activity in 1 – 8 cell embryos, but not in morula embryos. According to the expression of G6PDH, which catalyzes the first and irreversible step of PPP, it is probable that there exists pentose phosphate pathway in 1 – 8 cell embryos, but not in morula embryos.

Similarly Leese & Barton (1987) observed G6PDH

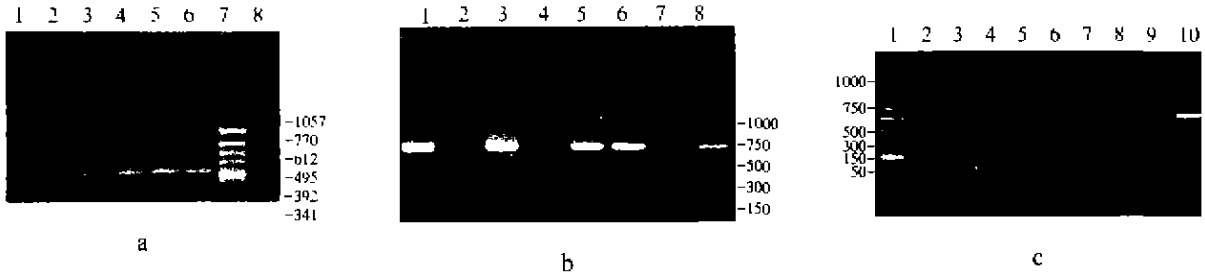

Fig.1 Nested, multiplex PCR for the pools of G6PDH, PFK and PGM mRNA from Kunming mouse embryos and liver (positive control)

a: 1.morula; 2.8-cell; 3.4-cell; 4.2-cell; 5.1-cell; 6.liver; 7.PCR markers; 8.negative control.

b: 1.liver; 2.negative control; 3.1-cell; 4.PCR markers; 5.2-cell; 6.4-cell; 7.8-cell; 8.morula.

c: 1.PCR markers; 2 – 3.1-cell; 4 – 5 2-cell; 6.4 – cell; 7.8-cell; 8.morula; 9.negative control; 10.liver.

activity, and found that stabilizes from the 1-cell stage up to the 8-cell stage and falls abruptly afterward in mice, whereas a more gradual decline was observed for human embryos (Martin *et al*., 1993). Decrease in G6PDH transcript do not find while development proceeds, but a significant temporary increase underlined at the 4-cell stage in bovine embryos (Lequarre *et al*., 1997). The down regulation seems to be due to a decrease in the number of molecules rather than to a change in kinetic properties (de Schepper *et al*., 1993). The levels of glucose metabolized are found to be low in mouse ovulated M Ⅱ oocytes, upon sperm entry, but PPP activity increases substantially (Urner & Sakkas, 1999). Pentose that is produced by PPP can be used as material to sythesize DNA and RNA in early embryos. It has been reported that the activity of the PPP increases threefold after 24 h of culture *in vivo* produced bovine embryos and is significatively higher

for poor quality ones (Javed *et al*., 1991).

2.2 PFK

Three PFK isozymes have been reported in mouse tissues, and those are liver-, muscle-and brain-type (Thrasher *et al*., 1981). Inner and outer pair primers were designed and synthesized according to the cDNA sequence of liver type phosphofructokinase (PFK) of mice. The nested reverse transcriptase-polymerase chain (Nested RT-PCR) was used to determine the transcription pattern of mRNA encoding PFK throughout 1-, 2-, 4-, 8-cell and morula mouse embryos. The results show that cDNA of PFK in mouse livers and 1 - 8 cell to morula embryos can be amplified an expected band using the inner pair primers by the templates of the products of the first PCR using the outer pair primers (Fig.2), and indicate that the transcription of PFK gene exists. PFK of early mouse embryos is possible the liver type.

PFK is the key enzymes for glycolysis and tricarboxylic acid cycle (TCA), so glycolysis and TCA is probable the important pattern of glucose metabolism in 1 - 8 cell and morula embryos.

In the preimplantation mouse embryo, PFK has been shown to be the primary point of a blockade to glycolysis (Barbehenn *et al*., 1974). This enzyme activity is turned off at the 2-cell stage and progressively activated at the following stages. Regardless of the presence or absence of glucose, it is a need to transfer embryos into fresh medium so as to increase blastocyst numbers and to reduce glycolytic metabolism (Leppens-Luisier & Sakkas, 1997). Glycolysis is the highest in the control bovine embryos and the lowest in the embryos cultured in protein-free medium. Both metabolism and blastocyst development of bovine embryos are altered by different culture media (Krisher *et al*., 1999). In this study, we have shown that PFK gene transcription was found in 1 - 8 cell to morula embryos; it indicated the activity of glycolysis is probable present in those embryos.

2.3 PGM

mRNA encoding PGM of mouse livers and 1 - 8 cell to morula embryos was detected by nested, muliplex PCR, and no transcription was seen at 1 - 8 cell to morula stages, except in the mouse liver (positive control, Fig.3).

PGM, an enzyme involved in glycogen metabolism, was 2000 times more active than glycogen phosphorylase at the 2-cell stage and fell progressively by the 8-cell stage, indicating that the embryo formed glycogen during the early stages but had difficulty using it because of insufficient glycogen phosphorylase (Hsieh *et al*., 1979). Brinster (1969) observed the similar results, but Ozias & Stern (1973) showed that the level of glycogen is low in mouse early embryos *in vivo* and glycogen metabolism is inhibited by some substances. We found that PGM gene do not transcript in mouse 1 - 8 cell to morula embryos. It indicated that there is no glycogenesis and glycogenolysis in the early embryos. Our results are not consistent with the others, which may be caused by the difference of mouse strains and methods for studies.

2.4 Conclusion

The transcription of G6PDH, PFK and PGM genes were detected by RT-PCR. G6PDH is present in 1 - 8 cell embryos, but not in morlae embryos. PFK presents in 1 - 8 cell to morula embryos. PGM is not present in the early embryos.

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昆明小鼠体内发育早期胚胎葡萄糖代谢关键酶转录产物的分析

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摘要：首次报道了昆明小鼠体内发育的早期胚胎1－细胞至桑椹期阶段葡萄糖代谢的3种关键酶——6－磷酸葡萄糖脱氢酶（G6PDH）、6－磷酸果糖激酶（PFK）和磷酸葡萄糖变位酶（PGM）的基因转录情况，其分别体现了磷酸戊糖、糖酵解、糖原的合成和分解等途径。根据G6PDH、PFK、PGM的cDNA序列分别设计和合成3套共6对内、外引物，采用巢式RT-PCR方法对其进行检测。结果表明：早期胚胎1～8细胞阶段均有G6PDH基因的转录，桑椹期胚胎不存在该基因的转录，说明早期胚胎1～8细胞阶段可能存在磷酸戊糖，而桑椹期则不存在；1－细胞至桑椹期均存在PFK基因的转录，说明该阶段的胚胎可能存在糖酵解代谢途径；1－细胞至桑椹期均不存在PGM基因的转录，说明该阶段的胚胎可能不存在糖原的合成与分解代谢途径。

关键词：昆明小鼠；胚胎；RT-PCR；基因转录；葡萄糖代谢

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